

# *Colletotrichum truncatum* Pathosystem on *Capsicum* spp: infection, colonization and defence mechanisms

N. P. Ranathunge · O. Mongkolporn · R. Ford ·  
P. W. J. Taylor

Received: 28 July 2011 / Accepted: 15 June 2012 / Published online: 9 July 2012  
© Australasian Plant Pathology Society Inc. 2012

**Abstract** The disease cycle of the chili (*Capsicum annuum*) anthracnose fungus *Colletotrichum truncatum* (formerly *C. capsici*) was elucidated from a study of infection and colonization of seed, leaves and fruit. Microscopic observations of detached leaves and fruit inoculated with a virulent pathotype (F83B), revealed direct cuticle penetration and, intramural, endophytic and necrotrophic phases of colonization. Seedling and fruit ripening stages were very susceptible to infection with the pathogen causing pre- and post-emergence damage and postharvest fruit rot. Furthermore, a quiescent stage, following leaf infection during the vegetative phase of plant growth served as a potential primary inoculum source for fruit infection. Leaf epidermal cells of the resistant *C. chinense* PBC932 expressed a strong hypersensitive response 48 h after infection (HAI) to both highly virulent (F83B) and less virulent (BRIP 26,974) pathotypes. Infected cells had thickened cell walls, cytoplasm aggregation, and high levels of reactive oxygen species produced 12 HAI. In contrast, the infected epidermal cells of the susceptible *C. annuum* cultivar Bangchang showed necrosis and rapid cell death after infection by either pathotype. Knowledge of the disease cycle of *C. truncatum* will be helpful in understanding the behaviour of

the pathogen in chili fields which will lead to more efficient application of control measures.

**Keywords** *Colletotrichum truncatum* · *Capsicum* species · Chili · Disease cycle

## Introduction

Anthracnose is a major disease of chili (*Capsicum* spp.) caused by a complex of *Colletotrichum* species (Than et al. 2008). Considered primarily as a fruit disease, anthracnose causes extensive yield losses and reduced marketability at the postharvest stage (Mahasuk et al. 2009a; Manandhar et al. 1995). *Colletotrichum truncatum*, formerly *C. capsici* (Damm et al. 2009) is one of the most destructive of the causal pathogens with multiple hosts (Pring et al. 1995) and several pathotypes (Mongkolporn et al. 2010; Montri et al. 2009), thus preventing the implementation of effective control measures.

A detailed understanding of the biology of the pathogen, host resistance and differential responses to pathotypes, as well as an understanding of the population genetic structures within and between growing regions is important for developing efficient and durable control measures. Recent microsatellite-based marker analysis of *C. truncatum* populations from chili growing regions in Southeast Asia revealed a high allele flow within and between populations, indicating a high adaptive potential for the pathogen to overcome host resistance and develop tolerance to fungicides (Ranathunge et al. 2009).

Advances in breeding for resistance to anthracnose have been limited by the lack of natural resistance within the *C. annuum* germplasm (Mahasuk et al. 2009a; Mahasuk et al. 2009b; Mongkolporn et al. 2010; Montri et al. 2009; Park 2007). However, resistance has been identified in the related *Capsicum* species, *C. chinense* and *C. baccatum* by the

---

N. P. Ranathunge  
Department of Agricultural Biology,  
Faculty of Agriculture, University of Ruhuna,  
Mapalana, Kamburupitiya 81100, Sri Lanka

O. Mongkolporn  
Department of Horticulture and Centre for Agricultural  
Biotechnology, Kasetsart University,  
Nakhon Pathom, Kamphaeng Saen 73140, Thailand

R. Ford · P. W. J. Taylor (✉)  
Melbourne School of Land and Environment,  
The University of Melbourne,  
Parkville, Victoria 3010, Australia  
e-mail: paulwjt@unimelb.edu.au

AVRDC (the World Vegetable Centre, Taiwan). The resistance derived from *C. chinense* PBC932 was differentially expressed at seedling and fruit stages. Three recessive genes *co1*, *co2* and *co3* were identified as being responsible for the resistance in mature green fruit (Pakdeevaporn et al. 2005), ripe fruit and seedlings (Mahasuk et al. 2009a), respectively. Although the mechanism of resistance is unknown, Mahasuk et al. (2009a) reported a hypersensitive reaction on leaves of PBC932 infected with *C. truncatum*; and Ko et al. (2005) identified a pepper esterase (*PepEST*) gene involved in resistance to *Colletotrichum* infection in chili fruit.

The potential to introgress resistance genes from related *Capsicum* spp. was recently affected by the discovery of pathotypes of *C. truncatum* that overcame resistance in *C. chinense* PBC932. Three *C. truncatum* pathotypes were identified on two differential genotypes of *C. chinense* including PBC932 and C04714 (Montri et al. 2009).

To better understand the mechanism of resistance in *Capsicum* spp, and virulence of *C. truncatum*, more detailed studies are required of the infection process and biochemical interactions that occur on the host in response to the invading pathogen. Depending on the species-host interactions, the initial host infection strategy for *Colletotrichum* species has been reported as either intracellular hemibiotrophic, subcuticular intramural necrotrophic or a combination of both strategies (Bailey et al. 1992). Although the infection mechanism of *C. truncatum* has been studied on cowpea (*Vigna unguiculata*), betel vine (*Piper betle*) and bean (*Phaseolus vulgaris*) by Pring et al. (1995) and on cotton by Roberts and Snow (1984), little is known about the biology and pathogenesis of *C. truncatum* on *Capsicum* species. Recently, Auyong et al. (2011) reported that *C. truncatum* colonized chili (*C. annuum*) fruit pericarp tissue intercellularly then entered an endophytic stage of its life cycle before the onset of necrotrophic infection. Kim et al. (2004) reported structural modifications and programmed cell death in chili in response to *C. gloeosporioides* infection.

The objectives of this paper were to study the infection and colonization processes involved in *Colletotrichum truncatum* infection of seeds, leaves and fruit of *Capsicum annuum* (chili); to elucidate important steps of the disease cycle of the pathogen; and to compare the cellular responses in leaf epidermal cells at the onset of infection in compatible and incompatible interactions between genotypes of *C. annuum* and *C. chinense*, and *C. truncatum* pathotypes in order to understand defence mechanisms employed by the host.

## Materials and methods

### Plant material

Plants of *Capsicum annuum* cv. Bangchang and *C. chinense* PBC932 were grown in 30 cm pots in the glasshouse at 25 °C.

Bangchang is highly susceptible and PBC932 is highly resistant to fruit infection by most isolates of *C. truncatum* (Montri et al. 2009; Pakdeevaporn et al. 2005).

### Isolates and inoculum preparation

Single spore isolates of *Colletotrichum truncatum*, F83B (isolated from *Capsicum annuum*, Western Thailand, virulent pathotype; Montri et al. 2009) and BRIP 26,974 (isolated from *C. frutescens* in Australia; low virulent pathotype; Taylor PWJ, unpublished) were maintained on potato dextrose agar (PDA; Difco, USA). For the inoculation experiments, subcultures were made on PDA and incubated for 7–10 days at 28 °C with a 12 h photoperiod until sporulation. Conidia were harvested by gently scraping the surface of the cultures using a sterile bent glass rod with 3 ml of sterile distilled water (SDW) and the suspension was filtered through six layers of muslin cloth. Spore concentrations were adjusted to the appropriate concentration using a haemocytometer and diluted with SDW.

### Initial infection process

Light microscopy was used to observe the initial infection process on whole leaf sections and on hand-sectioned fruit of cultivar Bangchang. For both experiments, the fruit and leaves were inoculated with  $10^6$  spores/ml of F83B isolate.

Surface sterilized, detached, 2nd and 3rd leaves from four-week-old plants were inoculated on the abaxial surface with  $6 \times 5$   $\mu$ l droplets of the inoculum and then were incubated in petri dishes containing sterile moist filter paper to maintain high relative humidity at 25 °C under dark conditions. Leaves were sampled at 6, 12, 24, 36, 48, 60 and 72 h after inoculation (HAI) for microscopic studies.

Fruit inoculation was carried out by placing  $3 \times 5$   $\mu$ l droplets on surface sterilized (1 % sodium hypochlorite for 10 min, washed in SDW twice) green (35 days after flowering; DAF) and red ripe (45 DAF) fruit. Inoculated fruit were kept in a humid chamber at 25 °C, under dark conditions for the first 48 h and then in a 12 h photoperiod.

### Sample preparation for light microscopic observation

Inoculated leaf pieces of approximately  $5 \times 5$  mm were cut using a scalpel and were placed in clearing solution of 1:2 absolute ethanol: glacial acetic acid for 24 h to remove chlorophyll (Curry et al. 2002). The clearing solution was then replaced with a fresh solution for another 12–24 h (Khan and Hsiang 2003). The same procedure was practiced for the hand sectioned inoculated fruit pieces. Hand sectioning for light microscopy observation was done at 24, 72 HAI and 7 days after inoculation (DAI) using a razor blade to obtain thin cross sections (approx. 0.5 mm) of inoculated fruit walls.

For microscopic observation, the cleared leaf and fruit tissues were stained in 0.05 % cotton blue (w/v) in lactophenol solution (20 % phenol, 20 % lactic acid, 40 % glycerol and 20 % water) (Ellis (2012) and destained by rinsing with 70 % ethanol and mounted on glass microscopic slides in clear lactophenol. The samples were examined using a Leica DMRBE light microscope and the images were captured by a Leica DC300F digital camera and Leica IM50 v.4 software.

#### Elucidation of the disease cycle of *C. truncatum*

**Seed Infection** *Capsicum annum* seeds were surface sterilized (as described above), and then transferred to 10 ml of either sterile water or a suspension of  $10^5$  or  $10^3$  spores/ml of the F83B isolate. Seeds were grown on moist sterile filter paper in Petri dishes (25 °C and a 12 h photoperiod) with five replicates and 25 seeds per replicate. The germination percentage and appearance of symptoms were observed daily for 7 days.

**Infection at post-emergence stage** The *C. annum* seedlings resulting from the inoculated ( $10^3$  spores/ml) and uninoculated seeds (25) were raised in sterile potting medium in seedling trays at 25 °C and a 12 h photoperiod. The appearance of symptoms at early stages of growth was observed. Surviving plant counts were taken daily. Three seedlings from each initial seed treatment were harvested, surface sterilized, and sections of the hypocotyls, cotyledons and root radicle were cultured on PDA at 3-day intervals. Fungal mycelium was subcultured on PDA and *C. truncatum* identified by colony and spore morphology according to the description given by Shenoy et al. (2007).

**Leaf infection at four-week-old plant stage** Healthy 4-week-old *C. annum* plants grown as previously described were spray inoculated with either  $10^6$  or  $10^3$  spores/ml of F83B isolate or with SDW. Six replicates were inoculated for each treatment. Subsequently, all leaves were tagged for future identification. The treated plants were kept at 100 % humidity for 24 h and then moved to a growth chamber at 25 °C in a 12 h photoperiod. At each weekly interval for 6 weeks, the two tagged upper most leaves of one plant per treatment were harvested and disease symptoms recorded. Then one leaf from each sample was prepared for microscopic observations (as indicated above), and the other surface sterilized and placed on PDA. Any fungal growth was subcultured on PDA and *C. truncatum* was identified as previously described.

**Stem infection at pre-flowering stage** Agar plugs (0.5 cm<sup>2</sup>) were cut from the leading hyphal edge of 7-day-old *C. truncatum* cultures (F83B) grown on PDA with a sterile cork borer. Seven-week-old healthy Bangchang plants, grown as previously described, were subsequently inoculated on the stem (~6 cm above soil level) by making two small wounds to a depth of ~1 mm with a sterile needle and by placing of the

agar plug on the wound area and sealing with parafilm®. Negative control plants were wounded and sealed in the same manner after placing sterile PDA agar plugs of the same size. Plants were observed for symptoms until 21 DAI. Stem tissue 10 cm above and 10 cm below the wound site was serially excised from four replicates per sampling into 2-cm pieces at 7, 14 and 21 DAI and cultured on PDA.

**Infection on flowering plants** Healthy, 8-week-old Bangchang plants containing 8–10 flower buds (green stage and petal opening stage; four replicates) were sprayed with either  $10^6$ ,  $10^4$  or  $10^3$  spores/ml of F83B isolate or SDW (for negative controls) and observed for the appearance of symptoms. The flowers were sampled at 4, 7 and 10 DAI and cultured in five replicates on PDA.

**Infection on fruit** Infection and colonization on green (35 DAF) and ripe (45 DAF) Bangchang fruit were assessed using the drop method of inoculation (Kanchana-udomkan et al. 2004) with three 5- $\mu$ l droplets of  $1 \times 10^6$  spores/ml of F83B isolate, placed on each fruit in five replicates. Negative controls were treated with SDW. The inoculated fruit were kept in a moist, humid chamber at 25 °C, under dark conditions for the first 48 h and then at 12 h photoperiod. Appearance of lesions was observed for 2 weeks.

**Test on seeds from infected fruit** Seeds from Bangchang infected fruit, from the previous experiment, were collected when >25 % of the fruit contained lesions (disease score 9 as described by Montri et al. (2009)) and were air dried on clean paper tissues at 25 °C for 2 days. For controls, seeds from five healthy fruit were extracted and air dried separately in the same manner. Seeds were surface sterilised (1 % sodium hypochlorite for 10 min, washed twice in SDW) and four replicates of 25 seeds per treatment were placed on moist filter paper (in sterile petri dishes) at 25 °C and a 12 h photoperiod to determine germination rates. Germinated seeds were transferred to steam sterilized soil and placed in a growth chamber at 25 °C and a 12 h photoperiod to detect appearance of symptoms on seedlings.

The data of above experiments were subjected to ANOVA procedure and means were compared using the Least Significant Difference (LSD) technique using statistical analysis system (sas 9.1) software (SAS institute Inc.).

#### Microscopic observation of differential interactions

Leaves and fruit of *C. annum* (Bangchang) and *C. chinense* (PBC932) genotypes were inoculated with  $10^6$  spores/ml of either the F83B or BRIP 26,974 *C. truncatum* isolates. Light microscopy was used to observe the differential host-pathogen responses during early disease development stages on leaves and fruit. Detached 2nd and 3rd leaves of four-week-old

Bangchang and PBC932 plants were inoculated and incubated as previously described. Four replications of  $\sim 0.75$  cm<sup>2</sup> leaf tissue segments from each treatment (host/pathogen combination) were excised using a sterile scalpel at 2, 6, 10, 12, 14, 24 and 48 HAI. The segments were then fixed and prepared for light microscopic observations as previously described. Three replicates were used for each host/pathogen combination.

#### Reactive oxygen species (ROS) generation in compatible and incompatible interactions

Inoculated detached leaves of *C. annuum* (Bangchang) and *C. chinense* (PBC932) genotypes treated with F83B or BRIP 26,974 isolates from the above experiment (four leaves from each treatment) were incubated in petri dishes containing sterile moist filter paper at 25 °C under dark conditions until sampled at 2, 4, 6, 8 and 10 HAI (4 h prior to the designated incubation period). Next, 2-cm<sup>2</sup> segments (in five replicates) were cut from each leaf and placed in 1 mg/ml 3,3'-diaminobenzidine (DAB)-HCl, pH 3.8 (Sigma-Aldrich, USA) and

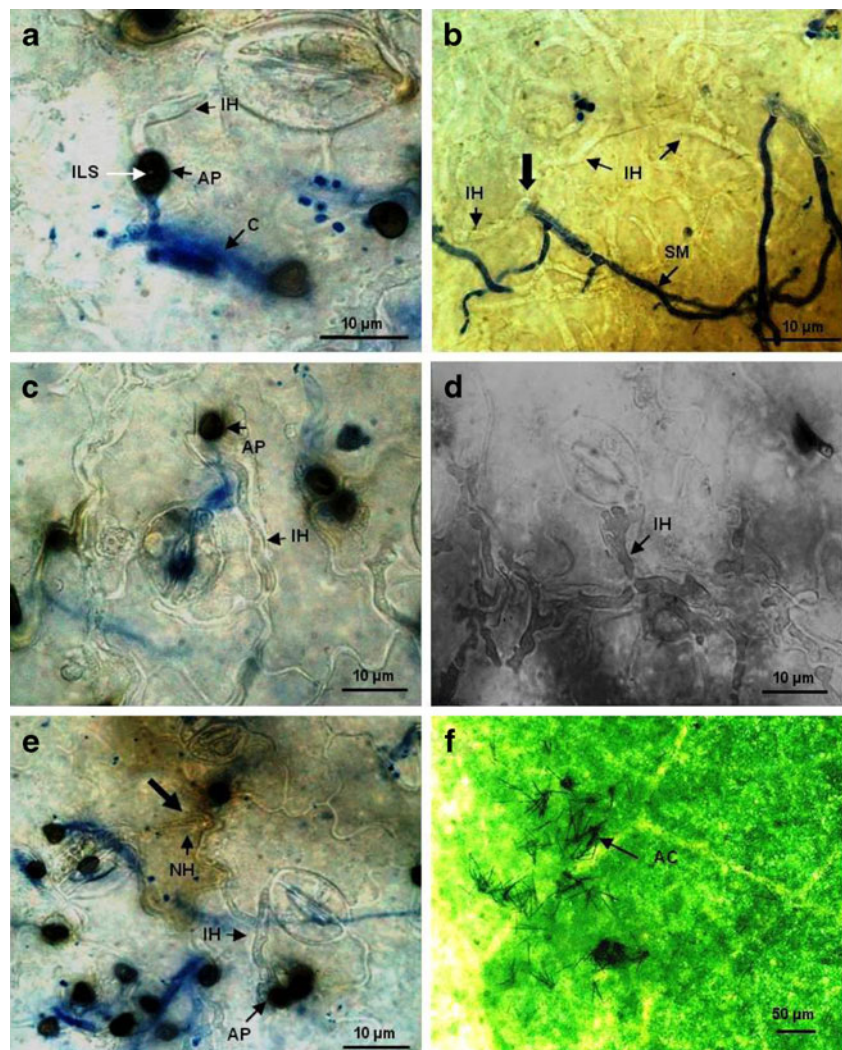
incubated at 25 °C in the dark (Shinogi et al. 2003; Thordal-Christensen et al. 1997) for 4 h (optimized time for the size of the leaf tissue) to complete the incubation. The samples were fixed, stained and mounted on a glass slide for light microscopic observation as previously described.

#### Results

##### Initial infection process -infection on detached leaves

On inoculated Bangchang leaves, conidial germination started as early as 2 HAI and by 12 HAI fully developed dark brown, melanised, globose to irregular shaped appressoria formed (Fig. 1a). Direct penetration of the cuticle by appressoria began from 12 to 24 HAI frequently near the cell junctions of the epidermis and was initially observed as an internal light spot (ILS) that appeared on the appressorium (Fig. 1a). Direct hyphal penetration was also observed from the surface mycelium at the later part of the infection process (72 HAI; Fig. 1b).

**Fig. 1** Penetration and colonisation of leaves of *C. annuum* cv Bangchang by *C. truncatum*. (a) Formation of appressoria, penetration peg (internal light spot) and infection hyphae. (b) Direct hyphal penetration at 72 HAI (block arrow indicate the penetration point) (c) Development of intramural hyphae beneath the cuticle at 48 HAI. (d) Extensive branching of intramural hyphae over the leaf tissue at 60 HAI. (e) Initiation of necrotrophic phase at 60 HAI (penetration of the necrotrophic hyphae marked by a block arrow). (f) Development of acervuli on inoculated leaves placed on water agar, 6 DAI. AP- appressorium, C- conidium, IH- intramural hypha, ILS- internal light spot, NH- necrotrophic hypha, AC – acervuli, SM- surface mycelium



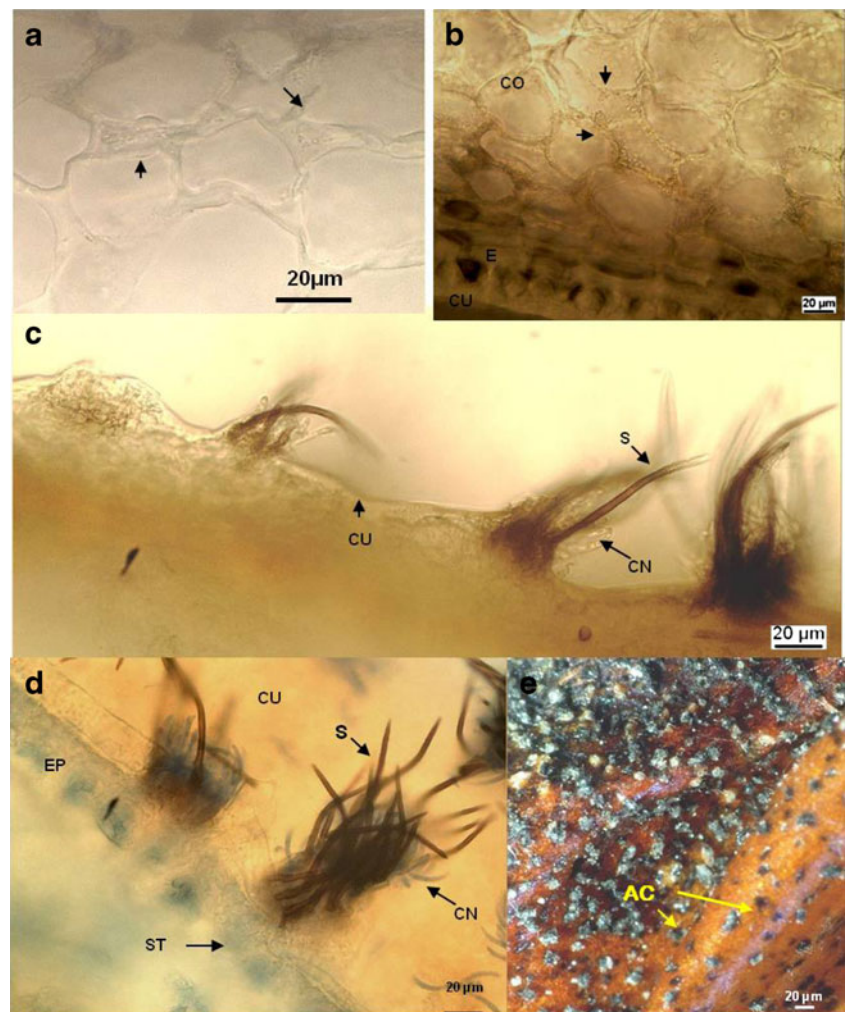
The colonization process was clearly observed at 48 HAI on inoculated leaves. A colourless primary infection hypha starting from an appressorium was observed which developed through the sub-cuticular layer and along the cell walls of the epidermis (Fig. 1c). The primary intramural hyphae colonized a large area of the tissue by branching profusely, yet were strictly limited to the cell walls (Fig. 1d). At this stage, the host cells did not show any sign of recognition of the fungal invasion since the underlying epidermal cells remained intact and turgid.

The necrotrophic phase of the infection began from 48 to 72 HAI where the intramural or primary hyphae gave rise to the secondary necrotrophic hyphae, which quickly penetrated epidermal and mesophyll cells causing extensive cell death within a short time (Fig. 1e). Resistance responses such as hypersensitive reaction and cytoplasmic aggregation were not observed in Bangchang leaf cells infected by F83B. The tissues of the detached leaves infected with F83B isolate totally disintegrated following 72 HAI, however these leaves maintained on water agar gave rise to acervuli by 6 DAI (Fig. 1f).

#### Initial infection process -infection on detached fruits

The time taken for F83B spore germination and appressoria formation on both ripe and green detached fruit was similar to that of detached leaves. Spores readily germinated soon after inoculation and produced appressoria with or without germ tubes. Penetration pores appeared from 24 to 48 HAI. However, symptoms did not appear at this stage of infection and the fruit cuticle remained intact and apparently undamaged even longer than on inoculated detached leaves that totally disintegrated at 72 HAI. At 4 DAI mycelia developed along the cell walls of the sub epidermal pericarp (Fig. 2a), however there was no visible cell damage and the hyphae appeared to be proceeding deeper into the fruit pericarp. By 6 DAI, dissolution of the sub epidermal collenchyma cell walls was observed due to mycelial colonization (Fig. 2b). Later, the affected tissues gave rise to the characteristic sunken appearance on the fruit surface. Despite the huge devastation to collenchyma tissue, the fruit cuticle and the adjoining epidermal cell layer remained undamaged and continuous along the surface.

**Fig. 2** Light micrographs showing the internal colonisation and the acervuli development of *C. truncatum* F83B isolate on red *C. annuum* fruits. Transverse sections of *C. annuum* red-fruit pericarp showing (a) Development of the fungal mycelium along the cell walls of sub-epidermal pericarp at 4 DAI; (b) Tissue destruction in collenchyma cells (CO) where arrowheads indicate the extensive damage to the cell walls and membranes; (c) Different stages of acervuli emergence through the cuticle. (d) A fully developed acervulus showing; CN- conidia stained in lactophenol-cotton blue, S- setae, ST- stroma of mycelium developed within the epidermal cell layer (EP). The cuticle (CU) remained intact in other areas. (e) Appearance of acervuli on fruit surface as seen through a stereo microscope. AC – acervuli



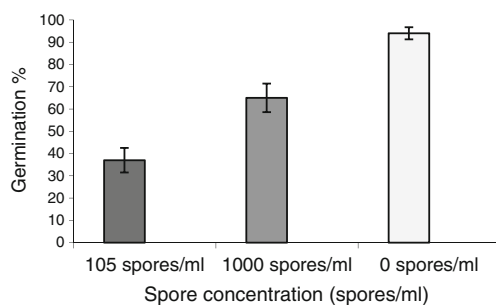
Emergence of acervuli on the inoculated fruit surface began by 7–9 DAI (Fig. 2c), and by this time, the fruit surface revealed prominent dark sunken characteristic anthracnose lesions which spread over the surface. The acervuli originated from a cushion of mycelia formed in epidermal layers of the pericarp close to the cuticle (Fig. 2d). The epidermis then ruptured to reveal the setae and conidia in tight clusters (Fig. 2e).

#### Elucidation of the disease cycle of *C. truncatum*

**Seed infection** There was a 60 % decrease in germination of Bangchang seeds when treated with F83B isolate at a spore concentration of  $10^5$  spores/ml at 7 DAI (Fig. 3). The seeds did not show any discolouration prior to germination though, a large number of appressoria appeared on the seed coats. Emerging roots of a few seeds were necrotic and died within a short time. Some seedlings died soon after emergence. At an inoculum concentration of  $10^3$  spores/ml, the germination rate decreased by 30 % compared to the uninoculated control seeds that gave rise to healthy seedlings (Fig. 3).

**Pathogenicity at post-emergence stage** By 3 DAI, only 64 % of the seedlings survived, which originated from the seeds treated with  $10^6$  spores/ml concentration of F83B isolate. The number of surviving seedlings decreased daily and by the 7th day after inoculation, only 12 % of the seedlings survived from the above treatment. When the spore concentration was reduced to 1,000 spores/ml, the survival rate was slightly increased even though only 28 % of the total seedlings raised managed to survive (Table 1).

The cotyledons of the transplanted Bangchang seedlings, which were recovered from inoculated seed appeared deformed, convoluted and remained entirely enclosed in the seed coat even after emergence compared to control plants with fully expanded cotyledons. In most of the treated seeds, cotyledons showed water-soaked necrotic lesions upon emergence from the seeds, and the seedlings died within



**Fig. 3** Percent seed germination of *Capsicum annuum* Bangchang in response to inoculation with different spore concentrations of the isolate F83B at 7 days after inoculation. Bars represent the standard error values

**Table 1** Number of *Capsicum annuum* cv. Bangchang seedlings (out of 25) survived after seed inoculation with *Colletotrichum truncatum* isolate F83B. DAI (days after inoculation). LSD values are at  $P=0.05$

	3DAI	4DAI	5DAI	6DAI	7DAI
Control	25 <sup>a</sup>	25 <sup>a</sup>	25 <sup>a</sup>	25 <sup>a</sup>	25 <sup>a</sup>
$10^6$ spores/ml	16 <sup>b</sup>	11 <sup>c</sup>	8 <sup>c</sup>	6 <sup>c</sup>	3 <sup>c</sup>
$10^3$ spores/ml	17 <sup>b</sup>	15 <sup>b</sup>	16 <sup>b</sup>	9 <sup>b</sup>	7 <sup>b</sup>

Values within a column followed by the same letter do not differ at  $P=0.05$

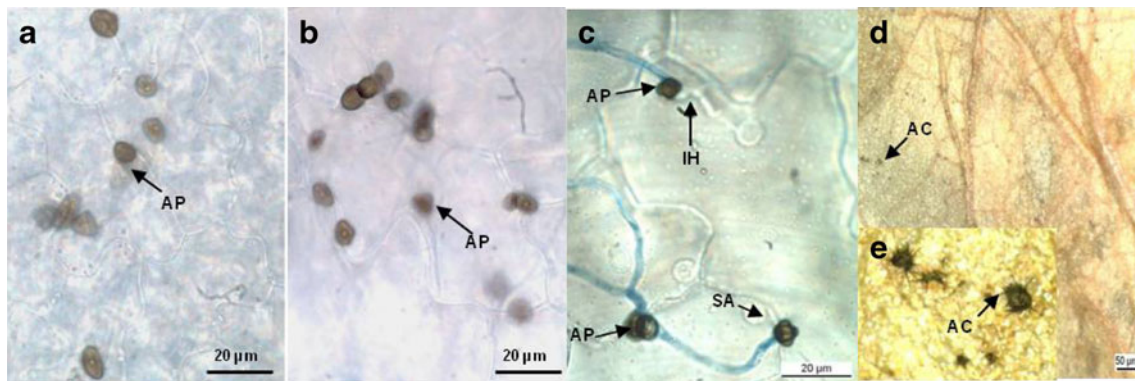
1–2 days of the first symptom appearance. The dead plants had developed a very short root system without branching when compared to control seedlings, but symptoms or pathogen colonization was not observed in the root system. Of the surface sterilized symptomless treated plants, *C. truncatum* was reisolated on PDA from the collar region, stem and from cotyledons.

**Leaf infection at 4-week-old plant stage** Inoculation of 4-week-old plants with a  $10^6$  spores/ml of F83B led to heavy defoliation, especially of the young leaves near the apex, by 72 HAI. Dark brown lesions were observed on leaves prior to defoliation. Presence of the pathogen was confirmed by culturing leaf tissues on PDA.

At  $10^3$  spores/ml, no plants died and symptoms did not develop on green leaves which remained healthy. Although plants continued to grow normally, light microscopy revealed the presence of appressoria attached to the inoculated leaf tissues (Fig. 4a and b). Although penetration pores developed on the appressoria from the first week after inoculation (WAI) there was no sign of subcuticular infection hyphae. When the inoculated leaves began to senesce (5–6 WAI), anthracnose lesions appeared on the leaf (Fig. 4d and e). Cleared tissues of yellowed leaves showed evidence of appressorial dormancy breakage, such as development of secondary appressoria and development of infection hyphae starting from previously quiescent appressoria (Fig. 4c). The falling leaves that were recovered and placed on water agar at 5 weeks after inoculation produced acervuli 3 days after culturing. Presence of the pathogen was confirmed by isolation on PDA from senescing leaves about 4–5 days after detachment from the plant.

**Stem infection at pre-flowering stage** Dark sunken lesions appeared at the point of inoculation at 7 DAI. Weekly serial culturing of the adjacent stem and nearby leaf tissues up to 21 DAI showed that the pathogen was confined to the first centimetre both above and below the inoculation point.

**Infection on flowering plants** Defoliation of leaves and flower drop occurred in the plants sprayed with spore



**Fig. 4** Light microscopic images of the *C. annuum* leaves sprayed *in planta* with F83B isolate. (a) 1 week after inoculation (WAI), (b) 4 WAI, (c) 5 WAI and (d and e) 6WAI. AP- appressorium, AC- acervuli, IH- infection hypha, SA – secondary appressorium

suspensions of  $10^6$  and  $10^4$  spores/ml of F83B. However, at  $10^3$  spores/ml, flower drop was reduced (Table 2) and there was no defoliation. By 72 HAI, necrosis was observed on dying flower buds leading to detachment of the flowers within 1 or 2 days. The corollas showed necrotic lesions and the unopened petals became convoluted. The unaffected flower buds continued to grow as normal and produced fruit. The pathogen was re-isolated from surface sterilized fallen flower buds but not from the symptomless flowers.

**Fruit infection** Drop inoculation of detached fruit showed that anthracnose lesion development on red-ripe fruit was faster than that on green fruit. Lesion development on red fruit started as small water-soaked patches by 5–6 DAI and expanded rapidly over the surface. By the time of 14 DAI acervuli that appeared abundantly on the fruit surface contained conidia (Fig. 2e). The infection of detached green fruit was delayed, however lesion development was observed by 14 DAI.

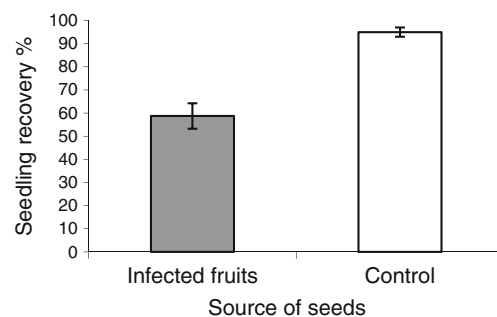
**Seeds from infected fruits** Acervuli were observed on seed coats of 10 % of the seeds from infected fruit. Radicles of these seeds died soon after emergence. The germination rate of the seeds from infected fruit was not significantly different to those of non infected fruit ( $P=0.05$ ). However, the transplanted healthy looking seedlings that originated from

infected fruit showed significant seedling death after 1 week when compared to non infected control seedlings (Fig. 5). The seedlings first looked healthy with few water soaked blotches on some of the cotyledons which eventually spread killing the seedling at very early stages.

#### Microscopic observation of differential interactions

For all combinations of the F83B and BRIP 26,974 isolates and the Bangchang and PBC932 host genotypes, conidial germination, appressoria formation and melanization, and appearance of penetration pores on leaves were observed within 24 HAI.

General necrosis was observed (around 48 HAI) following infection of leaves by both isolates in the Bangchang genotype, without any sign of resistance against the rapidly colonising pathogen. Cell death advanced quickly all over the tissue (Fig. 6a and b). In contrast, PBC932 appeared to express a much higher resistance to fungal infection (Fig. 6c and d). Penetration of the PBC932 leaf tissues by hyphae from appressoria of both isolates either through cuticle or through stomata led to rapid host response observed as a hypersensitive reaction. There appeared to be no opportunity for the



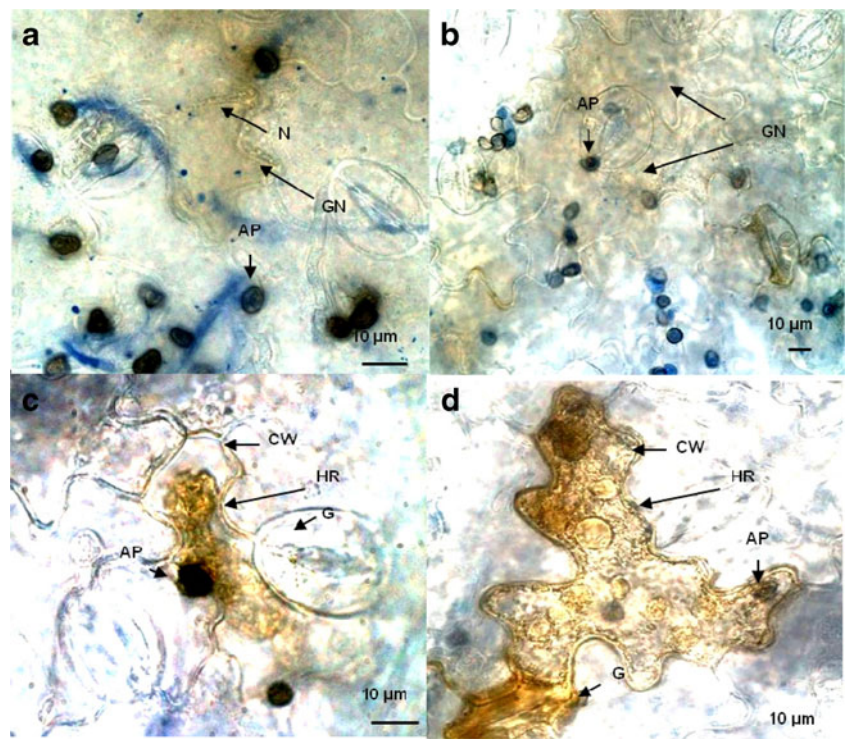
**Fig. 5** Percent recovery of seedlings 1 week after transplanting. The treatments were the seedlings obtained from germinated seeds extracted from infected fruits and non-treated control fruits. Bars represent standard error values

**Table 2** Effect of different spore concentrations of F83B on flower numbers of *Capsicum annuum* cv. Bangchang plants at flowering stage at 4, 7 and 10 days after inoculation. LSD values are at  $P=0.05$

	$10^6$ spores/ml	$10^4$ spores/ml	$10^3$ spores/ml	Control
4DAI	22.3 <sup>a</sup>	49.3 <sup>ab</sup>	67.0 <sup>b</sup>	88.3 <sup>c</sup>
7DAI	9.0 <sup>a</sup>	16.6 <sup>b</sup>	54.6 <sup>c</sup>	82.3 <sup>c</sup>
10DAI	5.0 <sup>a</sup>	11.0 <sup>a</sup>	53.0 <sup>b</sup>	70.3 <sup>b</sup>

Values within a row followed by the same letter do not differ at  $P=0.05$

**Fig. 6** Compatible (a and b) and incompatible (c and d) host-pathogen interactions between *Capsicum* genotypes and *Colletotrichum truncatum* pathotypes at 48 HAI. (a) Interaction between (Bangchang) and *C. truncatum* F83B isolate. (b) Interaction between *C. annuum* (Bangchang) and *C. truncatum* BRIP 26,974 isolate. (c) Interaction between *C. chinense* (PBC 932) and *C. truncatum* F83B isolate. (d) Interaction between *C. chinense* (PBC932) and *C. truncatum* BRIP 26,974 isolate. Note the clear structural differences in necrotic cells of the incompatible host (c and d), such as cell wall thickening and cytoplasm aggregation. AP- appressorium, CW- cell wall, G- guard cell, N- necrotrophic hypha, IH- intramural hypha, GN- general necrosis, HR – hypersensitive reaction



pathogen to colonise intramurally without being detected as was the case with Bangchang. The epidermal cells immediately below the penetration point and few other adjacent cells appeared necrotic, which revealed a quick recognition of the pathogen by the host. The necrotic cells showed characteristic features of hypersensitive (HR) cell death such as cytoplasmic aggregation and thickening of cell walls (Fig. 6c and d).

#### Reactive oxygen species (ROS) generation in compatible and incompatible interactions

Generation of reactive oxygen species was in accordance with the microscopic observations. The DAB stain deposited around the mature appressoria indicated the points of initial interaction. The reaction was observed in both compatible and incompatible interactions however, the stain was more prominent in the incompatible interaction indicating higher levels of reactive oxygen species expression (Fig. 7). The inoculated tissues tested for DAB deposition at 6, 8 and 10 HAI, did not result in a positive reaction. In all the combinations, DAB reaction was observed between 12 and 14 HAI.

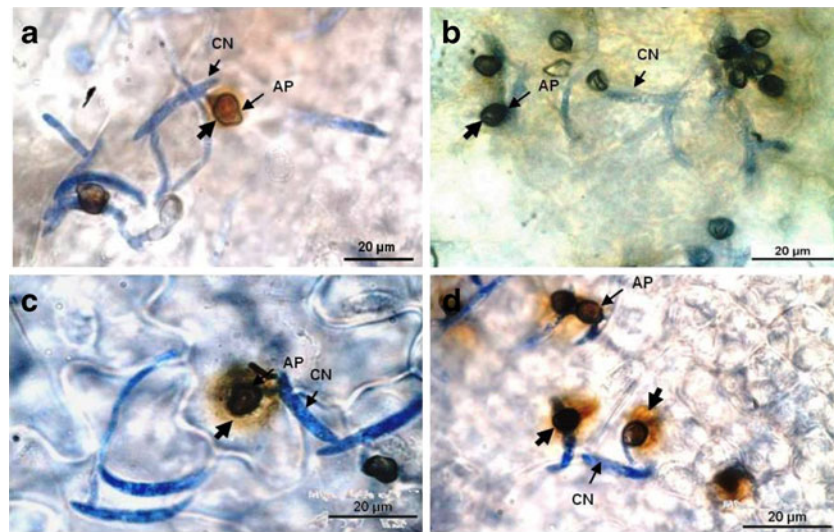
#### Discussion

Microscopic examination of *C. truncatum* inoculated leaves revealed the sequence of events that took place during the initial establishment of *Colletotrichum* infection. Pre-penetration

events on chili leaves from conidial germination to appressoria differentiation were similar to many other species of the *Colletotrichum* genus as reviewed by Bailey et al. (1992). The direct penetration through the cuticle appeared to be highly localized and the penetration pore was well defined indicating the possibility of secretion of cutin degrading enzymes during penetration (Dickman et al. 1982; Pring et al. 1995). The primary intramural hyphae colonized a large area of the tissue by branching profusely, yet was strictly limited to the cell walls. Auyong et al. (2011) also demonstrated that *C. truncatum* colonised chili fruit intramurally and entered a short endophytic life stage before infecting cells and causing necrosis. This feature of *C. truncatum* has given the fungus the advantage of infecting a wide range of hosts compared to relatively host specific hemibiotrophs (Pring et al. 1995). Switching from an intramural to a necrotrophic phase via an endophytic stage is another important aspect in *C. truncatum* infection, with each stage being dependent on the host genotype, environmental conditions, biochemical changes taking place during fruit ripening; and pathogen virulence factors (Auyong et al. 2011; Mongkolporn et al. 2010; Perfect et al. 1999).

The initial infection events were similar on both green and ripe Bangchang (susceptible genotype) fruit and the penetration started around 24–48 HAI, which was similar to leaf infection. The similarity of *Colletotrichum* spore germination and appressoria formation on both green and red-ripe chili fruit has been reported previously (e.g. Kim et al. 1999; Manandhar et al. 1995). As on leaves, the initial colonization of *C. truncatum* on chili fruit did not trigger





**Fig. 7** Light microscopic observations of Reactive Oxygen Species (ROS) generation in compatible and incompatible interactions between *Capsicum* spp. and *C. truncatum* pathotypes at 14 h after inoculation. (a) Compatible interaction between *C. annuum* vs. BRIP 6,974 isolate, (b) Compatible interaction between *C. annuum* vs. F83B isolate, (c)

Incompatible interaction between *C. chinense* vs. BRIP 6,974 isolate, (d) Incompatible interaction between *C. chinense* vs. F83B isolate. Bright coloured deposits around appressoria (indicated by arrows) show ROS generation in response to penetration. CN- conidium, AP- appressorium

either cell death or any apparent defence response until the initial symptoms appeared about 6 days after inoculation as small circular water-soaked patches near the inoculation site. This could probably be a latent phase of the infection process of *C. truncatum*, which is similar to quiescent phases of other *Colletotrichum* species that has been reported by many workers (e.g. Perfect et al. 1999) and the reported endophyte stage described by Auyong et al. (2011). Light microscopic observations revealed that, as soon as the symptom development started on both leaves and fruits, the internal cell dissolution was extensive irrespective of undamaged cuticle. This was similar to the observations by Pring et al. (1995) on cowpea hypocotyls. The growth of intramural hyphae was clearly evident within cell walls of collenchyma cells similar to that in leaf epidermal and mesophyll cells.

The key areas of the disease cycle of *C. truncatum* infecting capsicum were assessed using laboratory/glass-house-based bioassays and observation on natural field infection). Seed infection gave rise to seedling death which should result in considerable loss of plants at the nursery stage. Even though artificial inoculation with a high spore concentration caused high mortality, inoculation with a lower concentration showed a significant number of infected but healthy looking plantlets which grew like normal plants. This observation could be true for natural seed infection through diseased fruit since the seed recovered from anthracnose symptom bearing fruit produced 62 % viable seeds that produced entirely symptomless seedlings. Acervuli occurred on seeds under heavy infestation however, in general, most of the seeds recovered from diseased fruit did

not show any signs of the fungal infection. This may be a reason why seed borne infection occurs without being detected by the growers/traders. The seedlings germinated from infected seeds showed necrosis on the radicle soon after emergence and later on cotyledons, collar region and on hypocotyls, eventually killing the whole plant. The symptoms or the presence of the pathogen was not observed in underground parts even though the root length was considerably reduced in affected seedlings compared to uninfected seedlings.

Expression of anthracnose symptoms on *Capsicum annuum* Bangchang foliage infected by *Colletotrichum truncatum* appeared to be a function of inoculum density. Spore germination and appressoria formation *in planta* was as rapid as that was observed in the detached leaf bioassay however, the growth appeared to be suppressed soon after penetration. Since *C. annuum* has been shown to be totally compatible with this isolate of *C. truncatum* in detached leaf and fruit bioassays, this growth suppression could be a phenomenon occurring *in planta* for the benefit of the pathogen to remain quiescent until a later stage of fruit development that would be beneficial for the pathogen to complete its disease cycle. This hypothesis was further supported by appearance of anthracnose symptoms on leaves at leaf senescence. The green leaves appeared healthy except for a few water-soaked anthracnose-like symptoms that appeared within 24–48 HAI and remained symptomless until they naturally turned yellow at senescence. Biochemical changes that take place in the senescing leaves may act as a trigger for the dormant pathogen to resume growth, colonize tissue and produce asexual fruiting structures. This

revealed that the leaves (often symptomless) may serve as sources of primary inoculum for fruit infection. The infected fallen leaves bearing acervuli contained many spores and this has implications in the field for re-infection of leaves/fruit near the soil surface by means of rain splash. This may explain the dramatic appearance of anthracnose symptoms in chili fields after rainfall. Since *C. truncatum* spores appeared to be short-lived (unpublished) continuous rounds of infection and re-infection could be a requirement for the disease to manifest for longer periods.

Therefore, there is a clear guidance for the growers to implement hygiene practices in the field by removing plant debris and to concentrate on applying systemic fungicides during the vegetative stages of the crop which would control the quiescent infection where anthracnose disease is prevalent. At present, extensive amounts of fungicides are applied at later stages of fruit development only when the symptoms appear on fruit, which may be a reason for ineffective control of the disease.

When the stems of Bangchang plants at the pre-flowering stage were inoculated with *C. truncatum* F83B isolate, localized lesions appeared a week after inoculation. However, there was no evidence of external or internal progression of the infection within 3 WAI. It was more likely that the infection was strictly limited to the epidermis and cortical cell layers of the stems rather than spreading through the vascular system. In addition, the lesions on mature stems were highly localized with a very slow expansion rate over the surface, probably because the pathogen did not prefer mature stem tissues for colonization. Spray inoculation of flower buds showed that *C. truncatum* was capable of infecting the flowers of *Capsicum* spp. However, the flowers did not appear to be a suitable organ for the pathogen to infect and remain quiescent until fruit development because of heavy flower abortion. Nevertheless, flower abortion alone could lead to considerable yield loss.

No detectable differences were observed in the time taken for spore germination, appressoria formation and melanization, and appearance of penetration pores in both compatible and incompatible interactions, which probably indicated that there was no major interference for the pathogen to initiate pre-penetration events on either susceptible and resistant hosts. A similar observation was reported for *C. trifolii* infection on alfalfa cotyledons where the appressoria formed and matured on both susceptible and resistant hosts (O'Neill and Saunders 1994). However, a remarkable differentiation in host response to pathogen infection by the two interaction types occurred soon after penetration. The susceptible host, Bangchang, developed the symptoms faster and the infection led to general necrosis (observable to the naked eye) as early as 48 after inoculation. In contrast, the resistant host, PBC 932 recognized the pathogen (irrespective of the

degree of virulence) very quickly (within 36 HAI) after penetration. Hence, there was no detectable intramural phase in the infection process on the resistant host by either of the pathotypes, while on the susceptible host the intramural phase was very prominent and lasted for approximately 12–24 h. These observations suggested that the susceptible host lacked quick recognition of the invading pathogen irrespective of its virulence level. However, *C. chinense* showed very rapid recognition followed by a hypersensitive response, thereby avoiding extensive colonization by either pathotype. This observation strongly suggested the involvement of pathogenesis related gene expression by the resistant host upon penetration by *C. truncatum*. Further studies are required to understand the molecular basis of induction of the defence responses.

Expression of differential defence responses by the two different *Capsicum* species was further confirmed by detecting the generation of reactive oxygen species at the site of penetration. Higher levels of DAB deposition around the penetration site in *C. chinense* leaf tissues indicated the induction of a hypersensitive response.

To fully understand host reaction to pathotypes, the infection process on chili fruit needs to be studied. A major concern is the changes in susceptibility of the chili fruit during development (green/ripe red). The linkage between biochemical changes that take place during fruit development and potential changes in their susceptibility to anthracnose pathogens has been noted (Taylor PWJ, unpublished). In addition, Mahasuk et al. (2009b) reported that two different recessive genes were responsible for the resistances differentially expressed in mature green and ripe red fruit of *C. chinense* PBC932 infected by *C. truncatum*. Genetic studies of resistance to anthracnose in both *C. chinense* PBC932 and *C. baccatum* PBC80 exhibited the resistances differentially expressed at green and ripe fruit stages, which appeared to be controlled by different genes (Mahasuk et al. 2009a, b).

Furthermore, understanding of the structural defence mechanisms of resistant chili fruit using histological methods would also provide insights into successful breeding programs. Kim et al. (2004) reported that histological changes took place in a resistant variety of *Capsicum baccatum* against *Colletotrichum gloeosporioides*. The resistance mechanism(s) exploited using this knowledge may eventually be helpful in identifying and incorporating resistance genes through breeding programs. Hanania et al. (1999) reported that the timing of activation of the defence response after pathogen recognition is the most crucial factor for the success of host resistance. Therefore, the differential leaf responses may be used as a discriminative bioassay to identify temporal aspects of pathogen recognition in future tissue-specific differential gene expression studies.

**Acknowledgments** The authors wish to thank the PORES scholarship program of the University of Melbourne for partly funding this work and thank the Tropical Vegetable Research Center, Kasetsart University, Kamphaeng Saen Campus for field experiment support.

## References

- Auyong ASM, Ford R, Taylor PWJ (2011) Genetic transformation of *Colletotrichum truncatum* associated with anthracnose disease of chili by random insertional mutagenesis. *J Basic Microbiol* 51: doi:10.1002/jobm.201100250 (In press)
- Bailey JA, O'Connell RJ, Pring RJ, Nash C (1992) Infection strategies of *Colletotrichum* species. In: Bailey JA, Michael JJ (eds) *Colletotrichum: biology, pathology and control*. CAB International, Oxon, pp 88–120
- Curry KJ, Abril M, Avant JB, Smith BJ (2002) Strawberry anthracnose: histopathology of *Colletotrichum acutatum* and *C. fragariae*. *Phytopath* 92:1055–1063
- Damm U, Woudenberg JHC, Cannon PF, Crous PW (2009) *Colletotrichum* species with curved conidia from herbaceous hosts. *Fungal Divers* 39:45–87
- Dickman MB, Patil SS, Kolattukudy PE (1982) Purification, characterization and role in infection of an extracellular cutinolytic enzyme from *Colletotrichum gloeosporioides* Penz on *Carica papaya* L. *Physiol. Plant Pathol* 20:333–347
- Ellis D (2012) [http://www.mycology.adelaide.edu.au/Laboratory\\_Methods/Microscopy\\_Techniques\\_and\\_Stains/lactophenol.html](http://www.mycology.adelaide.edu.au/Laboratory_Methods/Microscopy_Techniques_and_Stains/lactophenol.html)
- Hanania U, Furman-Matarasso N, Ron M, Avni A (1999) Isolation of a novel SUMO protein from tomato that suppresses EIX-induced cell death. *Plant J* 19:533–541
- Kanchana-udomkan C, Taylor PWJ, Mongkolporn O (2004) Development of a bioassay to study anthracnose infection of chilli pepper fruit caused by *Colletotrichum capsici*. *Thai J Agric Sci* 37:293–297
- Khan A, Hsiang T (2003) The infection process of *Colletotrichum graminicola* and relative aggressiveness on four turfgrass species. *Can J Microbiol* 49:433–442
- Kim KD, Oh BJ, Yang J (1999) Differential interactions of a *Colletotrichum gloeosporioides* isolate with green and red pepper fruits. *Phytoparasitica* 27:1–10
- Kim KH, Yoon JB, Park HG, Park EW, Kim YH (2004) Structural modifications and programmed cell death of chilli pepper fruit related to resistance responses to *Colletotrichum gloeosporioides* infection. *Phytopath* 94:1295–1304
- Ko MK, Jeon WB, Kim KS, Lee HH, Seo HH, KimYS Oh BJ (2005) A *Colletotrichum gloeosporioides*-induced esterase gene of non-climacteric pepper (*Capsicum annum*) fruit during ripening plays a role in resistance against fungal infection. *Plant Mol Biol* 58:529–541
- Mahasuk P, Khumpeng N, Wasee S, Taylor PWJ, Mongkolporn O (2009a) Inheritance of resistance to anthracnose (*Colletotrichum capsici*) at seedling and fruiting stages in chilli pepper (*Capsicum* spp.). *Plant Breed* 128:701–706
- Mahasuk P, Taylor PWJ, Mongkolporn O (2009b) Identification of two new genes conferring resistance to *Colletotrichum acutatum* in *Capsicum baccatum* L. *Phytopath* 99:1100–1104
- Manandhar JB, Hartman GL, Wang TC (1995) Anthracnose development on pepper fruits inoculated with *Colletotrichum gloeosporioides*. *Plant Dis* 79:380–383
- Mongkolporn O, Montri P, Supakaew T, Taylor PWJ (2010) Differential reactions on mature green and ripe chilli pepper fruit infected by three *Colletotrichum* species. *Plant Dis* 94:306–310
- Montri P, Taylor PWJ, Mongkolporn O (2009) Pathotypes of *Colletotrichum capsici*, the causal agent of chilli pepper anthracnose, in Thailand. *Plant Dis* 93:17–20
- O'Neill NR, Saunders JA (1994) Compatible and incompatible responses in alfalfa cotyledons to races 1 and 2 of *Colletotrichum trifolii*. *Phytopath* 84:283–287
- Pakdeevaporn P, Wasee S, Taylor PWJ, Mongkolporn O (2005) Inheritance of resistance to anthracnose caused by *Colletotrichum capsici* in *Capsicum*. *Plant Breed* 124:206–208
- Park HG (2007) Problems of anthracnose in pepper and prospects for its management. In: The First International Symposium on Chilli pepper Anthracnose, Convention Center. Seoul National University, Korea, Page 19
- Perfect S, Hughes HB, O'Connell RJ, Green JR (1999) *Colletotrichum*: a model genus for studies in pathology and fungal-plant interactions. *Fungal Genet Biol* 27:186–198
- Pring RJ, Nash C, Zakaria M, Bailey JA (1995) Infection process and host range of *Colletotrichum capsici*. *Physiol Mol Plant Pathol* 46:137–152
- Ranathunge NP, Ford R, Taylor PWJ (2009) Development and optimization of sequence-tagged microsatellite site markers to detect genetic diversity within *Colletotrichum capsici*, a causal agent of chilli pepper anthracnose disease. *Mol Ecol Resour* 9:1175–1179
- Roberts RG, Snow JP (1984) Histopathology of cotton ball rot caused by *Colletotrichum capsici*. *Phytopath* 74:390–397
- Shenoy BD, Jeewon R, Lam WH, Bhat DJ, Than PP, Taylor PWJ, Hyde KD (2007) Morpho-molecular characterisation and epitypification of *Colletotrichum capsici* (Glomerellaceae, Sordariomycetes), the causative agent of anthracnose in chilli pepper. *Fungal Divers* 27:197–211
- Shinogi T, Suzuki T, Narusaka Y, Park P (2003) Microscopic detection of reactive oxygen species generation in the compatible and incompatible interactions of *Alternaria alternata* Japanese pear pathotype and host plants. *J Gen Plant Pathol* 69:7–16
- Than PP, Jeewon R, Hyde KD, Pongsupasamit S, Mongkolporn O, Taylor PWJ (2008) Characterization and pathogenicity of *Colletotrichum* species associated with anthracnose on chilli pepper (*Capsicum* spp.) in Thailand. *Plant Pathol* 57:562–572
- Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H<sub>2</sub>O<sub>2</sub> in plants: H<sub>2</sub>O<sub>2</sub> accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J* 11:1187–1194